The Chemistry of Vitamin B_{12}. Part 19.[1] Labilisation of the Cobalt–Carbon Bond in Organocobalamins by Steric Distortions; Neopentylcobalamin as a Model for Labilisation of the Vitamin B_{12} Coenzymes

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Neopentylcobalamin in neutral solution at 25 °C is stable under nitrogen but is decomposed by O_2 and by imidazole to give a cob(III)alamin and by hydrogen-atom donors such as thiols and Pr^iOH to give the cob(II)alamin and neopentane. The reaction is ascribed to reversible homolytic fission of the Co–C bond to give a low steady-state concentration of the cob(II)alamin and neopentyl radicals, which can then form neopentane by the abstraction of a hydrogen atom. Neopentylcobinamide is also decomposed by high concentrations of imidazole, while the purely five-co-ordinate neopentylcobalamin in acid and neopentylcobinamide in neutral solution are stable both in the presence and absence of O_2. Labilisation of the Co–C bond is ascribed to steric distortion around the co-ordinated C atom in the six-co-ordinate neopentyl complexes. It is suggested that neopentylcobalamin provides a model for the labilisation of the Co–C bond in the vitamin B_{12} coenzymes, and possible mechanisms for the isomerase reactions are discussed.

ENZYMES containing the so-called coenzyme derivatives of vitamin B_{12}, which all possess the 5′-deoxy-5′-adenosyl ligand (see Figure 1, Part 17),[2] catalyse various isomerisations involving the 1,2-interchange of a H atom with a C atom [in $-COSR$, $-CHNH_2COOH$ and $-C(=CH_2)COOH$], NH_2 or OH group.[3,4] In spite of considerable experimental work and much speculation, the mechanisms of these reactions have not been established.

The main items of experimental evidence for the mechanism can be summarised as follows. (i) Electronic and e.s.r. spectra show that the Co–C bond undergoes reversible fission to give a cobalt(II) corrinoid and an organic radical, which may be derived from either the ligand or the substrate. (ii) Labelling experiments show that the migrating H atom of the substrate (designated SH) exchanges with the H atoms on C_α [C(5′)] of the ligand (designated R), but not with the solvent. (iii) There is also some evidence for the intermediate formation of deoxyadenosine (RH).[3,4] Speculation centres on the mechanism of cleavage of the Co–C bond in the coenzyme [to give cobalt-(I), -(II), or -(III) corrinoids] and the form in which the substrate actually undergoes isomerisation (radical, carbonium ion, or ligand on Co). The simplest possible pathways[5] are shown in Figure 1; more complex schemes can be written for substrates which undergo a shift of NH_2 or OH.

As already pointed out,[2] experiments with synthetic analogues of the coenzyme have shown[6,7] that the mechanism of cleavage of the Co–C bond does not require the presence of any heteroatom on the ligand, at least not closer than the adenine N situated five atoms away from the Co atom; yet bonds between Co and a primary alkyl group without a functional atom are usually very stable.[8] We have suggested[5] that the protein may (by a conformational change on binding the substrate) distort the co-ordination sphere of the Co (most likely by distorting the Co–C–C bond angle), thereby displacing equilibrium (1) to the right, and we have therefore studied the effects of steric distortion in simple alkyl and cycloalkyl ligands containing no functional groups, on the spectra, equilibria, and reactions of organocobalamins. We have shown that increasing the degree of substitution or distortion on either C_α or C_β leads to parallel changes in spectra and equilibrium constants (*e.g.* to an increase in the pK value) of organocobalamins and have concluded that the main variable, as seen by the Co atom and the ligands, is an increase in the Co–C bond length.[2] We have also reported the first example of the isomerisation of an

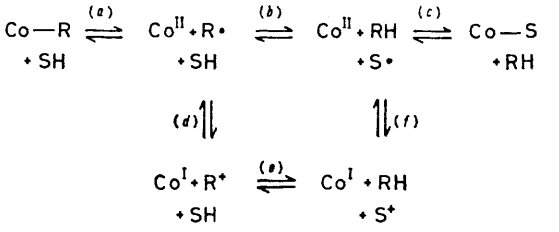

FIGURE 1 Schematic representation of the simplest possible pathways (ref. 5) for the vitamin B_{12}-dependent isomerase reactions, where the coenzyme and its ligand are denoted by Co–R and the substrate and its transferable hydrogen atom by SH

organo-ligand without irreversible cleavage of the Co–C bond (*viz.* cyclopropylcarbinyl to allylcarbinyl) in a preliminary communication.[9] We report here our results on the effects of steric distortion on the labilisation of the Co–C bond with particular reference to the reactions of neopentylcobalamin, which emerged as the most likely candidate to provide a model for the labilisation of the Co–C bond in the coenzymes.†

$$Co\text{–}R \rightleftharpoons Co^{II} + R^{\cdot} \qquad (1)$$

Two different mechanisms have now been established for the unassisted decomposition of organocorrinoids with simple alkyl ligands possessing no functional groups, *viz.* homolytic fission to give the cobalt(II) corrinoid and

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a free radical according to (1), and the synchronous elimination of Co from C_α and H from C_β to give an olefin and the cobalt(I) corrinoid or an equivalent complex; these provide the background to the present studies.

Homolytic fission has been most thoroughly studied in the case of the photolysis of methylcobalamin. The acceleration of the rate of photolysis by the presence of various reagents which react with free radicals (*e.g.* O_2, thiols, alcohols, or quinones), the nature of the products formed, and a comparison of the kinetics under N_2 and O_2,[8,10] together with the recent direct study of the reverse formation of methylcobalamin [11,12] are all consistent with an initial reversible fission of the Co–C bond according to (1) where R = Me, followed by irreversible reaction of the methyl radical with O_2, *etc.* Thermal fission has been less well studied since it requires higher temperatures (see Table 1), but it appears to be analogous to photolysis; *cf.* the isomerisation of methylcorrinoids on heating and under the influence of light.[13]

The instability of isopropylcobalamin and the catalysis by B_{12s} of the decomposition of Pr^iBr to propylene and HBr were first reported by us in 1968.[14-16] The reverse reaction of propylene (and of other simple olefins) with B_{12s} in non-aqueous solvents [*e.g.* $MeCO_2H$ or MeOH–$MeCO_2H$ (1 : 1)] to give isopropylcobalamin was reported by Schrauzer and Holland in 1971.[17] These (dark) reactions obviously represent a second class of reversible and unassisted reactions of organocorrinoids. Grate and Schrauzer [18] have recently studied them in more detail and shown that the rate of decomposition of isopropylcobalamin in aqueous solution is independent of pH (above the p*K* of the cobalamin) and of the presence or absence of O_2 (in contrast to homolytic fission), and shows an isotope effect which indicates that the β-H is eliminated in the rate-determining step, and gives B_{12s} (reaction with MeI gives methylcobalamin in virtually stoicheiometric yield) and propylene (stoicheiometry not determined). They also found a parallel between the variation in rates for the decomposition of cycloalkylamine oxides (a known *syn*-elimination reaction) and the cycloalkylcobalamins, *viz.* $C_8 > C_7 > C_5 > C_6$ in both cases, and concluded that the mechanism involves the concerted (or *syn*-) β-elimination of an olefin with transfer of the H atom to the Co. We accept the concerted β-elimination mechanism but suggest that, in view of the known reactivity of the corrin ring [8,19] and the known occurrence of addition reactions which involve the Co atom and the methine C atom in other cobalt complexes,[20] it is more likely that the H atom is transferred to an atom [probably C(5), C(10), or C(15)] of the corrin ring; the exact mechanism is, however, immaterial to the discussion below.

EXPERIMENTAL

Materials.—Samples of vitamin B_{12} and B_{12a} were kindly given by Mr. A. P. Domleo of Glaxo–Allenbury (Pty) Limited, South Africa. The organocobalamins and neopentylcobinamide were prepared as previously described.[2] Imidazole (Aldrich, 99%) was recrystallised three times from benzene. Pinacol (B.D.H. laboratory reagent) was recrystallised from diethyl ether. Isopropyl alcohol and n-propanol (both Merck, for analysis), cysteine hydrochloride (Merck, 99%), thioglycolic acid (B.D.H. laboratory reagent, 97%) and neopentane (Matheson, C.P.) were used as received.

Methods.—Ultraviolet–visible spectra were recorded with a Unicam SP8000 or a JASCO UVIDEC-1 spectrophotometer, both fitted with a variable-temperature unit; all spectra were recorded in 1-cm cells and, unless otherwise stated, at 25 °C. Thin-layer chromatography (t.l.c.) was carried out with Merck silica gel (60 F-254) on cellulose coated plates.

Gas chromatography (g.c.) was carried out on a Packard 427 gas chromatograph with a 1.5 m × 4 mm column of 5% silicone SE 52 on Anakrom at 60 °C with helium as carrier gas and a flame-ionisation detector. The reaction mixtures to be analysed were contained in a 500-cm^3 flask fitted with a rubber diaphragm seal, and samples of the gas phase were withdrawn with a micro-syringe for analysis every 30—60 min. Solutions consisted of (*a*) neopentylcobalamin (400 cm^3 of 6×10^{-5} mol dm^{-3}), Pr^iOH (4.9 mol dm^{-3}), and phosphate buffer pH 7.1; (*b*) neopentylcobalamin (300 cm^3 of 1.7×10^{-4} mol dm^{-3}), thioglycolate (0.15 mol dm^{-3}), and phosphate buffer pH 5.6; (*c*) neopentylcobalamin (300 cm^3 of 6×10^{-5} mol dm^{-3}) and phosphate buffer pH 7.2; (*d*) Pr^iOH (400 cm^3 of 4.9 mol dm^{-3}) and phosphate buffer pH 7.1; and (*e*) thioglycolate (300 cm^3 of 0.15 mol dm^{-3}) and phosphate buffer pH 5.6. All solutions were thoroughly de-oxygenated before mixing. Solution (*c*) was thermostatted at 60 °C, the other solutions kept at room temperature.

RESULTS

The rates of decomposition of *ca.* 3×10^{-5} mol dm^{-3} solutions of various organocobalamins were studied by u.v.–visible spectrophotometry (scanning the range 300—650 nm) with the various ligands and under the different conditions of pH, temperature, and gas phase (N_2 or air) shown in Table 1. At pH 1 all the organocobalamins are present as the yellow five-co-ordinate ' base-off ' (E) forms, while at pH 6 they are present as a temperature-dependent mixture of the red six-co-ordinate ' base-on ' (A) forms and the yellow five-co-ordinate ' base-off ' ($C + C^{dbzm}$) forms; for further details see ref. 1. The spectra of neopentylcobalamin at pH 6.8, which consists of *ca.* 60% of the A and 40% of the C form, and at pH 1 are shown in Figure 2.

For all the reactions of Table 1 under nitrogen the main product was identified from the position of the main absorption bands [8] and their disappearance on admitting air as B_{12r}, in either the ' base-on ' or ' base-off ' form at pH 6—7 and 1 respectively. In each reaction the changes in spectra produced a semblance of isosbestic points and the changes in optical density (at 473 and 470 nm for the two forms of B_{12r}) suggested the formation of 60—100% B_{12r}. After admitting air to these reaction mixtures the spectra revealed the presence of varying amounts of ' stable yellow corrinoids,' characterised by the presence of a band at *ca.* 465 nm which is unaffected by light and air but shifted to 485 nm on adding cyanide.[8] The overlap between the absorption band of B_{12r} and of the stable yellow corrinoid prevented any decision as to whether some or all of the stable yellow corrinoid was formed during decomposition under

nitrogen or only after the admission of air, and also prevented any reliable determination of the yield of B_{12r}; the absence of good isosbestic points indicated the occurrence of some side reaction(s) even under nitrogen. It has recently been shown that the cobalamins of Pr^i, C_5, and C_6 decompose by β-elimination to give B_{12s} and the olefin as

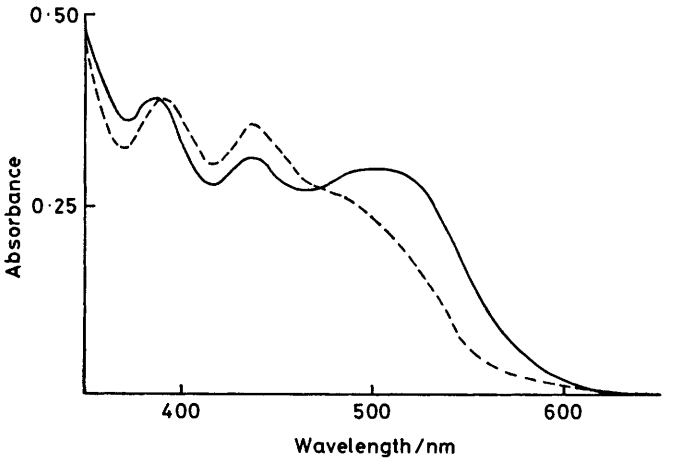

FIGURE 2 Spectra of 3.7×10^{-5} mol dm^{-3} solutions of neopentylcobalamin in phosphate buffer pH 6.8 (——) and in 0.1N H_2SO_4 (– – – –)

the primary products; [18] the apparent formation of B_{12r} at pH 1 and 6, without any increase in absorption around 386 nm (the main band of B_{12s} [8]), can be ascribed to the known decomposition of B_{12s} to B_{12r} with liberation of H_2 in acid and neutral solutions (C_3, C_4, C_5, and C_6 denote cyclopropyl, -butyl, -pentyl, and -hexyl respectively).[8]

Reactions in the presence of air produced spectra corresponding to the formation of aquocobalamin (band at 350 nm) in 80—100% yield (as calculated from the change in optical density at 350 nm), together with a more pronounced shoulder at *ca.* 460 nm, which is again ascribed to the formation of stable yellow corrinoids, and the semblance of isosbestic points.

The results of Table 1 indicate that both the cyclohexyl and neopentyl (np) cobalamins may be of potential interest as models for labilisation of the Co–C bond in the coenzymes towards homolytic fission. Although cyclohexylcobalamin decomposes under nitrogen by β-elimination, the fact that the rate of decomposition is accelerated by the presence of air indicates that homolytic fission according to (1) can also occur at room temperature in competition with β-elimination. Neopentylcobalamin, on the other hand, is stable under nitrogen but decomposed by air. The possible occurrence of homolytic fission at room temperature can, therefore, be studied without complications due to the simultaneous occurrence of β-elimination in the case of neopentylcobalamin.

Neopentylcobalamin was tested for possible (dark) reactions with various reagents under the conditions shown in Table 2. The corrinoid products were identified from their u.v.–visible spectra (see above). Neopentylcobalamin is also decomposed by white light (tungsten lamp) in the absence of added reagents to give B_{12r}. Photolysis was therefore used, in the case of the slower reactions, in order to complete the decomposition and obtain the final spectrum for determining the rate of decomposition.

The reactions of neopentylcobalamin with cysteine and thioglycolate both produced spectra which indicated the formation of 65% (average of two experiments with 62 and 69%) and 53% B_{12r} respectively, as calculated from the final optical density at 473 nm. The appearance of weak bands at 370 nm in both cases indicated the formation of some of the thiolatocob(III)alamin (*cf.* refs. 21—23), probably due to traces of O_2. Superposition of the successive spectra only gave an approximation to isosbestic points. All three alcohols produced spectra with a clear maximum at 473 nm, due to the formation of B_{12r} (65 and 67% yield in the case of Pr^iOH), and reasonable isosbestic points were observed.

It is difficult to prove that B_{12r} is the primary product in these reactions because (i) B_{12s} decomposes to B_{12r} and H_2 at a rate which increases as the pH falls,[8] while (ii) thiols reduce B_{12a} to B_{12r} at a rate which increases as the pH rises.[23] Further experiments with 3×10^{-5} mol dm^{-3} cobalt showed (iii) that the presence of 1 mol dm^{-3} Pr^iOH decomposes B_{12s} to B_{12r}, decomposes tetrahydroborate, and hence also prevents the reduction of B_{12r} to B_{12s} by tetrahydroborate at pH *ca.* 9, but does not reduce B_{12a} to B_{12r}. It appeared impossible to find a combination of thiol con-

TABLE 1

Rates of decomposition [a] of organocobalamins

pH	Gas phase	(θ_c/°C)	Alkyl ligand: Me	C_3	Et	C_4	Bu^i	C_6	Pr^i	C_5	np
1	N_2	25	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
		50	n.d.	n.d.	n.d.	n.d.		≤5%	20%	36%	
		80	n.d.	n.d.	n.d.	7%		25 min	7 min	5 min	
1	Air	25	n.d.	n.d.	n.d.	n.d.					n.d.
		50	n.d.	n.d.	n.d.	3%					
		80	≤5%	≤5%	10%	23%					
6—7	N_2	25	n.d.	n.d.	n.d.	n.d.	n.d.	*ca.* 1 h	<2 min	<1 min	n.d.
		50					21%				24% [d]
6—7	Air	25	n.d.	n.d.	n.d.	n.d.	n.d.	21 min [b]	*c*	<1 min	60 min [e]
		50	n.d.	n.d.	n.d.	n.d.	21%				
		80	n.d.	n.d.	30 min	10%					

[a] All solutions contained *ca.* 3×10^{-5} mol dm^{-3} cobalamin in 0.1N H_2SO_4 or, for the neutral solution, in either phosphate buffer pH 6.4—6.9 (C_3, C_6, Pr^i, np) or unbuffered deionised water pH *ca.* 6 (all others). Where decomposition is observed, the rate is given as either (*i*) the half-life of reaction or (*ii*) the percent decomposed in one hour; n.d. denotes no detectable (≤2%) decomposition in 1 h. [b] $t_{\frac{1}{2}}$ = 20 and 22 min in two separate experiments. [c] Grate and Schrauzer (ref. 18) have shown that the presence of air has no effect on the rate of decomposition of isopropylcobalamin at pH 7. [d] 23 and 24% decomposition in 1 h in two separate experiments. [e] $t_{\frac{1}{2}}$ = 55, 60, and 60 min in three separate experiments.

centration and pH which allowed the reaction of neopentylcobalamin with thiols to proceed at a reasonable rate and other reactions at only negligible rates. The observed formation of B_{12r} therefore strongly suggests that this is the primary product in the reaction of neopentylcobalamin with both thiols and alcohols, but this evidence alone cannot rigorously exclude the intermediate formation of B_{12s} in the case of alcohols or of either B_{12s} or a cob(III)-alamin in the case of the thiols.

The presence of thiols (and other reducing agents such as hydroquinone) accelerates the oxidation of B_{12r} by air, and this has been ascribed to the reaction of the transient O_2 adduct of B_{12r} with these reducing agents.[24] Although Pr^{i}OH behaves similarly to thiols in decomposing neopentylcobalamin, the presence of 1 mol dm^{-3} Pr^{i}OH has no noticeable affect on the rate of oxidation of 3×10^{-5} mol dm^{-3} B_{12r} under our conditions ($t_{\frac{1}{2}}$ *ca.* 5 min both with and without Pr^{i}OH) or on the amount of stable yellow corrinoids formed after admitting air, provided the reaction mixture is not allowed to stand too long. It was, however, noticed that the longer the reaction mixture was allowed to stand under nitrogen (with no further detectable change in the spectrum), the greater the amount of stable yellow corrinoids formed on admitting air. A reaction mixture containing 6×10^{-5} mol dm^{-3} neopentylcobalamin and 4.9 mol dm^{-3} Pr^{i}OH was allowed to stand for 22 h and, after the admission of air, phenol–chloroform extraction, and concentration, was examined by t.l.c. on cellulose using a Bu^{s}OH–H_2O–acetic acid (100 : 50 : 1) mixture.[25] This showed the presence of a fast-moving yellow spot (R_F 0.35) and a yellow streak or series of spots (R_F 0.07—0.28) with no trace of B_{12a} (R_F 0.07) or any other red spot; all the yellow spots appeared to be stable to light, which again supports their identification as stable yellow corrinoids. The occurrence of these reactions may complicate attempts to analyse the reaction mixture for organic products.

Gas chromatography of samples taken at 30—60 min intervals from the gas phase above the reaction mixture in two experiments each with Pr^{i}OH and thioglycolate (see Experimental section for details) revealed the presence of a single peak with the same retention time (84 s) as neopentane, which showed a regular increase in intensity before levelling off after *ca.* 7 and 3 h respectively with the two reagents. The same peak was also observed when neopentylcobalamin was heated alone at 60 °C and pH 7.2 under N_2 for 2 h, but not obtained from Pr^{i}OH or thioglycolate alone at 25 °C for 3 h under N_2. The g.c. trace of thioglycolic acid alone contained a large number of indistinct peaks which prevented the detection of any disulphide which might have been formed from the thiol.

The 2-hydroxypropyl radical, which is expected to be formed by reaction of the np radical with Pr^{i}OH, may undergo several reactions. Pinacol has been identified as a product (from dimerisation of the radical) [26] and evidence has been presented for the formation of acetone (from oxidation of the radical by B_{12r}) [12] in the photolysis of organocobalamins in the presence of Pr^{i}OH, *i.e.* under conditions of relatively high radical concentrations. The g.c. trace from the gas phase above the Pr^{i}OH reaction mixture showed no peaks corresponding to acetone or any other detectable products. After standing overnight the Pr^{i}OH reaction mixture was concentrated by rotary evaporation and extracted with diethyl ether; t.l.c. of the ether extract on silica using chloroform–methanol (10 : 1) [26] revealed two spots (R_F 0.08 and 0.77) which did not correspond to pinacol (R_F 0.60) and were not identified. It appears that neither acetone nor pinacol is produced under our conditions, although complications due to the reactions observed on admitting air cannot be excluded.

The reaction of neopentylcobalamin with imidazole proceeds in (at least) two stages. The final spectrum is typical of a red cobalt(III) corrinoid with α, β, and γ bands at 538, 508, and 357 nm respectively and can be assigned to imidazole–cobalamin since B_{12a} reacts ' instantaneously ' to give the same bands under the same conditions (*cf.* ref. 27). The intermediate also has a band at *ca.* 508 nm but far less absorption around 540 nm and a less pronounced shoulder at *ca.* 410 nm; it has not been identified. The first stage gives isosbestic points at 374 and 490 nm and follows reasonable pseudo-first-order kinetics with $t_{\frac{1}{2}} = 5$ min. The overall reaction, as followed at 538 nm, gives $t_{\frac{1}{2}} = 15$—20 min. Neopentylcobinamide also reacts with imidazole to give a similar final spectrum with bands at 538, 508, and 357 nm, which can be assigned to bis(imidazole)cobinamide (*cf.* ref. 28) and also proceeds in two stages; the overall reaction, as followed at 538 nm, shows 27, 30, and 35% (average 31%) change in 1 h.

TABLE 2

Reactions of neopentyl-cobalamin and -cobinamide

Complex	pH	Reagent [a]	Main product	Rate of reaction [b]
Neopentylcobalamin (base-on)	6.8	None	n.d.	(n.d. over 4 h)
	6.8	Air saturated	B_{12a}	55, 60, 60 min
	6.8	Cysteine (0.028 mol dm^{-3})	B_{12r}	3.5 h (14%)
	6.7	Thioglycolate (0.04 mol dm^{-3})	B_{12r}	20 min
	7.2	Pr^{i}OH (3.3 mol dm^{-3})	B_{12r}	60 min (50%)
	7.1	Pr^{n}OH (3.3 mol dm^{-3})	B_{12r}	15%
	9.2	Imidazole (1.3 mol dm^{-3})	Co^{III} [c]	5 min [d]
Neopentylcobalamin (base-off)	1.0	Air saturated	n.d.	n.d.
	2.2	Cysteine (0.1 mol dm^{-3})	n.d.	n.d.
	1.3	Pr^{i}OH (1.3 mol dm^{-3})	n.d.	n.d.
Neopentylcobinamide	6.7	None	n.d.	n.d.
	6.7	Air saturated	n.d.	n.d.
	6.7	Pr^{i}OH (3.3 mol dm^{-3})	n.d.	n.d.
	8.4	Imidazole (6 mol dm^{-3})	Co^{III} [e]	*ca.* 30% [d]

[a] All solutions contained *ca.* 3×10^{-5} mol dm^{-3} Co and were studied under N_2 (unless otherwise stated) at 25 °C. [b] Where decomposition is observed, the rate is given as either (*i*) the half-life of reaction or (*ii*) the percent decomposed in 1 h; n.d. denotes no-detectable (≤ 2%) decomposition in 1 h. [c] Imidazole–cobalamin. [d] See text. [e] Bis(imidazole)cobinamide.

DISCUSSION

The data of Table 1 show that, amongst the organocobalamins studied here, only those of Pr^i, C_5, C_6, and np in neutral solution show any significant reactivity at room temperature. The first three decompose even under nitrogen, and the mechanism has recently been shown to involve β-elimination to give B_{12s} and the olefin.[18] For reasons to be given below, β-elimination is probably not relevant to the mechanism of action of the coenzyme. We therefore focus attention on neopentylcobalamin, which appears to be the only simple, unsubstituted alkylcobalamin so far reported which is stable under nitrogen but decomposed by O_2. The decomposition of neopentylcobalamin in the presence of air has already been noted,[2,18] but without any comment on the mechanistic implications. Evidence for homolytic fission of the Co–C bond to give B_{12r} and np radicals according to equation (1) is provided by a comparison of the nature of the reagents which decompose neopentylcobalamin, their relative rates of reaction, and the nature of the products with analogous results on the reactions of the methyl and adenosyl radicals which are formed by the photolysis of methylcobalamin and dbc (vitamin B_{12} coenzyme).

It is known that the photolysis of methylcobalamin is reversible and accelerated by the presence of reagents which react with the methyl radical and/or B_{12r} and so drive equilibrium (1) to the right.[8,10] If the accelerating effect of these reagents were linearly related to their concentration, then comparison under the same conditions of concentration should give the following order of accelerating effect: $O_2 \gg$ homocysteine > benzoquinone $\gg Pr^iOH > Pr^nOH > EtOH > MeOH$.[8] The presence of O_2 produces B_{12a} and a variety of organic products;[8] cysteine, homocysteine, and 1,4-naphthaquinone produce *S*-methylcysteine, methionine, and 2-methyl-1,4-naphthaquinone [26,29] respectively, but the valency of the Co has not been reported. It was assumed that the methyl radicals abstract H atoms from the alcohols to give methane, but this was not established experimentally.[26] The photolysis of dbc is not reversible because of the rapid cyclisation of the free radical, and the rates of photolysis are virtually the same in air and under nitrogen, probably because the rate-determining step is the actual photolysis;[8,30] the nature of the products can, however, be changed by the addition of certain reagents, obviously through competition for the initially formed free radical. The free radical will react with O_2 to give adenosine-5-carbaldehyde,[8] with homocysteine to give *S*-adenosylhomocysteine,[31] with other thiols,[32-34] and with Pr^iOH [34] to give deoxyadenosine. The presence of O_2 leads to the formation of B_{12a}, but the valency of the Co has not been reported in the other cases. In the absence of added reagents, the free radical cyclises onto the *meso*-carbon atom of the imidazole ring of the adenine and B_{12r} is formed in stoicheiometric yield.[29] Photolysis of 2,2′-isopropylidene-5′-deoxyuridinylcobalamin under nitrogen, however, leads to cyclisation with concomitant reduction of the heterocyclic ring and the formation of B_{12a}. The methyl and adenosyl radicals can, therefore, initially either (i) abstract a H atom (*e.g.* from Pr^iOH and thiols) to give methane and deoxyadenosine, or (ii) add to unsaturated compounds (heterocycles and quinones) to give methyl and adenosyl derivatives; the nature of the subsequent steps has not been established.

The present results on neopentylcobalamin have shown (i) that neopentylcobalamin is decomposed in neutral solution by O_2, by thiols (cysteine and thioglycolate), by imidazole and by certain alcohols (Pr^iOH and Pr^nOH), (ii) that, if the rates of reaction are converted to a common concentration, then they fall in the order O_2 > thioglycolate > cysteine ~ imidazole > $Pr^iOH > Pr^nOH$, (iii) that both thioglycolate and Pr^iOH produce neopentane, and (iv) that thiols and alcohols produce B_{12r}, while O_2 and imidazole produce cob(III)alamins. There is obviously a very close parallel with the results obtained from the photolysis of methylcobalamin and dbc; we have not investigated the possible effect of quinones. The attack by imidazole parallels the cyclisation of the adenosyl radical, while the further oxidation to the Co^{III} state parallels the reaction of the uridinyl analogue of dbc. Comparison of the relative rates of reaction of the cobalamin and cobinamide with imidazole (Table 2) and of the relative equilibrium constants for the co-ordination of dbzm (60% base-on) and imidazole ($\log K < -1.5$) [2] demonstrates that the reaction shown by neopentylcobalamin with imidazole must be ascribed to the ' base-on ' cobalamin and not to any np–Co–imidazole complex or ' base-off ' form.

We conclude that the neopentylcobalamin reacts *via* an initially reversible and homolytic fission of the Co–C bond according to (1) to give B_{12r} and the np radical, which can then abstract a H atom from thiols and alcohols to give the hydrocarbon or undergo other reactions with O_2 and imidazole. Both the u.v.–visible spectrum of neopentylcobalamin (Figure 1), which shows no absorption in the 550—700 nm region typical of B_{12r},[8] and the very slow rate of decomposition under nitrogen (Table 1) indicate that the concentration of B_{12r} and free radicals at equilibrium is low, but sufficient to enable reactions of the free radical to be observed at room temperature. Our results demonstrate that the ligand in neopentylcobalamin can undergo the same types of reaction (*e.g.* with thiols and Pr^iOH) at room temperature which the ligand in protein-free dbc can only undergo on photolysis.

We have also shown (Table 1) that the five-co-ordinate acidified (E) form of neopentylcobalamin is stable towards O_2, cysteine, and Pr^iOH and that the five-co-ordinate neopentylcobinamide in neutral solution is stable towards Pr^iOH and O_2 (other reagents not tested). The reactions of neopentylcobalamin at pH 6 can therefore be ascribed to the 60% which is present as the six-co-ordinate, base-on (A) form.[1] A comparison of the effects of dbzm and imidazole as axial ligands, required in order to test the effect of a change in corrin conformation [1] on reactivity, is complicated by

the role of imidazole as reagent as well as potential ligand and by the very low formation constant for co-ordination of imidazole. Since, however, we can ascribe the faster reaction observed between neopentylcobalamin and imidazole to the ' base-on ' cobalamin (see above), the much slower reaction observed for neopentylcobinamide with imidazole must be due to a reaction involving either the np–Co–imidazole complex or the five-co-ordinate neopentylcobinamide itself; since the five-co-ordinate neopentylcorrinoids are inert towards other reagents (see above), we conclude that the reaction of neopentylcobinamide with imidazole requires the transient formation of the six-co-ordinate imidazole complex. The evidence shows that the six-co-ordinate neopentylcorrinoids (with either dbzm or imidazole as ligand) are very much more labile than the five-co-ordinate neopentylcorrinoids (whether cobinamides or acidified cobalamins).

Cyclohexylcobalamin in neutral solution decomposes faster in air than under N_2 (Table 1) which suggests that it can decompose by homolytic fission as well as by β-elimination and that the homolytic fission is reversible. Comparison of $t_{\frac{1}{2}}$ in air and N_2 indicates that homolytic fission in air alone would give $t_{\frac{1}{2}}$ *ca.* 30 min, which is similar to that of neopentylcobalamin ($t_{\frac{1}{2}}$ *ca.* 60 min). The isopropyl and cyclopentyl complexes could presumably also undergo homolytic fission, but this is obscured by the faster rate of β-elimination. Acidification to give the five-co-ordinate (E) form inhibits both homolytic fission and β-elimination at room temperature (Table 1).

We have previously used the spectra and pK values of organocobalamins to establish the order of ligands $Me \sim C_3 \sim R_{dbc} < Et \sim Pr < Bu^i \sim C_4 < Pr^i \sim C_5 \sim C_6 \sim np$ (R_{dbc} = the alkyl ligand present in the coenzyme derivatives), and shown that there is no difference between the effects of increasing and decreasing Co–C_α–C_β (*cf.* np and C_6).[2] We have associated this order with an increase in the length, and hence presumably a decrease in the strength, of the Co–C bond. This, in turn, should show some correlation with lability, provided there are no major changes in the relative stabilities of the free radicals produced; *cf.* the decrease and increase respectively in conformational energy on forming the cyclopentyl and cyclohexyl radicals.[35] It is, therefore, gratifying to find that the cobalamins which undergo homolytic fission most readily in air at pH 6 include those of C_6 and np (and do not necessarily exclude those of Pr^i and C_5). The data of Table 1 also show that the rates of decomposition in air at pH 1 increase in the order $Me \sim C_3 < Et < C_4$, which again agrees with the previously established order of ligands. From this rough correlation and from the greater lability of the six- over the five-co-ordinate forms (where the Co is probably displaced out of the equatorial plane towards the apical ligand atom) [1] we conclude that the origin of lability [*i.e.* of the displacement of equilibrium (1) to the right] is distortion of the bond angles around C_α with a concomitant increase in the Co–C_α bond length.

It is worth noting that other metal–neopentyl complexes,[36] including the cobaloxime,[37] are relatively inert. It appears that only the combination of the bulky np (and not even Bu^i) with the corrin ring and its upward-projecting substituents can provide the necessary degree of steric repulsion and leverage to distort the bond angles and bond lengths around C_α.

Lability towards β-elimination shows a similar variation. Reactivity is highest with ligands such as Pr^i, C_5, and C_6 (see Table 1 and ref. 18) and, at least in the case of the isopropyl and cyclohexyl corrinoids, far higher in the six-co-ordinate base-on cobalamin and imidazole adduct of the cobinamide than in the five-co-ordinate cobinamide and acidified cobalamin.[18,38] Here again, increased lability can be ascribed to an increase in steric distortion around C_α and in the Co–C_α bond length and probably also to placing the β-H atom closer to a methine C atom of the corrin ring. Grate and Schrauzer [18] have attributed the greater lability of the ' base-on ' over the ' base-off ' forms to a change in the conformation of the corrin ring; their conclusion is, however, vitiated by the similar effects of dbzm and imidazole.

Possible mechanisms for the isomerase reaction (see Figure 1) can now be discussed. As before,[5] we shall assume (i) that the only atoms on either the protein or the coenzyme which take part in redox reactions or the making and breaking of covalent bonds are Co and C_α [C(5′)], and (ii) that there is some common denominator in the mechanisms of all the isomerase reactions. The two key questions concern (i) the mechanism of ' activation ' of the Co–C bond in the coenzymes and (ii) the form in which the substrate undergoes rearrangement.

Homolytic fission and β-elimination are both potentially available pathways for the ligand in the coenzymes. Schrauzer combines our discovery of the reaction of N_2O with B_{12s} [39] with his observation that N_2O inhibits the enzyme to conclude that the initial step must involve β-elimination to give B_{12s}; [40] even allowing that N_2O can react only with B_{12s}, his observations could equally well be explained by the formation of B_{12s} at some later stage (see Figure 1 and below). He also admits that ' it is particularly difficult to formulate a mechanism for the . . . conversion of succinyl–CoA into methylmalonyl–CoA.' [40] Since there is no model for the reaction of B_{12s} with substrates such as glutamate, β-elimination will not be discussed further.

Our results on neopentylcobalamin show that if the Co–C bond to a primary alkyl ligand is subjected to strain, equilibrium (1) can be displaced sufficiently far to the right to allow the formation of a steady-state concentration of free radicals even at room temperature; and we suggest that neopentylcobalamin provides a good model for the *principle* by which the protein labilises the Co–C bond in the coenzymes. The protein must, however, be able to displace the equilibrium much further than is observed in neopentylcobalamin, especially in the case of coenzymes such as adenylcobamide coenzyme, which is normally five-co-ordinate even in

neutral solution.[22] We have pointed out that a conformational change in the protein can bring about a very large change in an equilibrium constant, which is not limited by the free-energy change for the binding of the substrate.[5] Since both increasing and decreasing the $Co-C_\alpha-C_\beta$ bond angle (*cf.* np and C_6) are 'seen' as changes in the Co–C bond length,[2] it appears that the type of strain (bending and stretching) engendered by the protein is not critical and presumably varies considerably from one enzyme or substrate to another.

The transfer of a hydrogen atom or hydride ion from a C–H bond to an organic radical or carbonium ion [steps (*b*) and (*e*) respectively] is well known. A good model for the electron transfer in steps (*d*) and (*f*) is the extremely fast (k_2 *ca.* 4×10^9 dm^3 mol^{-1} s^{-1}, but temperature apparently not stated) reduction of B_{12r} by the 2-hydroxypropyl radical Me_2COH to give B_{12s} and the carbonium ion, which then loses a proton to give acetone.[12,41] Our observation that air accelerates the decomposition of cyclohexylcobalamin, which implies a reversible homolytic fission, appears to be the first evidence that normal secondary, as well as primary, alkyl radicals can react with a cobalt(II) corrinoid to form a Co–C bond, as required by step (*c*). Adequate models are therefore available for all the steps in Figure 1.

Since 1975 Dowd *et al.*[42,43] and Rétey and coworkers [44,45] have discovered several examples of carbon skeleton rearrangements analogous to those observed in the enzymatic reactions, when enzymatic substrates or closely related derivatives are placed as ligands on the Co atom in cobalamins and cobaloximes, and the Co–C bond irreversibly broken, usually by photolysis. Rétey has also shown that the free radical alone will not give rearranged products in the absence of the cobalt and has used an ingenious method to provide evidence that the yield of the isomerised product increases as the residence time of the free radical in the neighbourhood of the Co^{II} ion is increased. He concludes that 'rearrangement of the . . . substrate radical is to all probability catalysed by the central cobalt atom'.[45] In 1976 we reported the isomerisation of cyclopropylmethyl- to but-3-enylcobalamin without irreversible cleavage of the Co–C bond;[9] *cf.* also refs. 46 and 47. In subsequent work we have been unable to detect any spontaneous isomerisation of a *secondary* to a *primary* organocobalamin using ligands such as Pr^i, Bu^s, and succinyl derivatives.[48] All the evidence suggests that at least the carbon skeleton rearrangements require the combination of S with the cobalt(II) complex and cannot occur as S· alone or as Co–S while it retains an intact Co–C bond. The simplest explanation for the role of the cobalt(II) complex is to promote the transient formation of the carbonium ion S^+ according to step (f). It remains to be seen whether other rearrangements also require a cobalt(II) complex.

The mechanism which we proposed [5] for the isomerase reactions incorporated the generally accepted idea of an initial homolytic fission of the Co–C bond in the coenzyme, but introduced new features regarding both the initial step and the isomerisation step. We suggested that the role of the protein is to displace equilibrium (1) to the right by distorting the co-ordination sphere of the cobalt (most probably the $Co-C_\alpha-C_\beta$ bond angle), and that this occurs when the substrate is bound at the active site and induces a change in the conformation of the protein; our present results on neopentylcobalamin provide strong support for these ideas. We also suggested that at least some substrates (*e.g.* glutamate and β-methylaspartate) might undergo rearrangement as the carbonium ion, formed according to steps (*b*) + (*f*) or, less likely, (*d*) + (*e*); and that, if all the isomerase reactions shared the same mechanism, then this was most likely to involve carbonium ions. Halpern [49] has independently suggested a carbonium-ion mechanism for the ethanolamine ammonia-lyase reaction. The evidence now available points in this direction. Other relevant aspects of the interaction between cobalt(II) corrinoids and S· radicals will be discussed elsewhere.[50]

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REFERENCES

[1] Part 18, S. M. Chemaly and J. M. Pratt, preceding paper.
[2] Part 17, S. M. Chemaly and J. M. Pratt, *J.C.S. Dalton*, 1980, 2259.
[3] B. M. Babior, *Accounts Chem. Res.*, 1975, **8**, 376.
[4] R. H. Abeles and D. Dolphin, *Accounts Chem. Res.*, 1976, **9**, 114.
[5] J. M. Pratt, in 'Topics and Techniques in Bioinorganic Chemistry,' ed. C. A. McAuliffe, Macmillan, London, 1975, p. 109.
[6] R. H. Abeles, *Adv. Chem. Ser.*, 1971, **100**, 346.
[7] H. P. C. Hogenkamp, paper presented at the 3rd European Symposium on Vitamin B_{12} and Intrinsic Factor, Zürich, 1979.
[8] J. M. Pratt, 'Inorganic Chemistry of Vitamin B_{12},' Academic Press, London, 1972.
[9] S. M. Chemaly and J. M. Pratt, *J.C.S. Chem. Comm.*, 1976, 988.
[10] J. M. Pratt and B. R. D. Whitear, *J. Chem. Soc.* (*A*), 1971, 252.
[11] J. F. Endicott and G. J. Ferraudi, *J. Amer. Chem. Soc.*, 1977, **99**, 243.
[12] J. F. Endicott and T. L. Netzel, *J. Amer. Chem. Soc.*, 1979, **101**, 4000.
[13] W. Friedrich and R. Messerschmidt, *Z. Naturforsch.*, 1969, **B24**, 465.
[14] R. A. Firth, H. A. O. Hill, B. E. Mann, J. M. Pratt, R. G. Thorp, and R. J. P. Williams, *J. Chem. Soc.* (*A*), 1968, 2419.
[15] H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, Abstract B1, I.U.P.A.C. 5th International Symposium on Natural Products, London, 1968.
[16] H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *Chem. in Britain*, 1969, 156.
[17] G. N. Schrauzer and R. J. Holland, *J. Amer. Chem. Soc.*, 1971, **93**, 4060.
[18] J. H. Grate and G. N. Schrauzer, *J. Amer. Chem. Soc.*, 1979, **101**, 4601.
[19] B. Grüning and A. Gossauer, paper presented at the 3rd European Symposium on Vitamin B_{12} and Intrinsic Factor, Zürich, 1979; B. Grüning, Dissertation, Braunschweig, 1979.
[20] M. G. Weiss and V. L. Goedken, *J. Amer. Chem. Soc.*, 1976, **98**, 3389.
[21] N. Adler, T. Medwick, and T. J. Poznanski, *J. Amer. Chem. Soc.*, 1966, **88**, 5018.
[22] H. A. O. Hill, J. M. Pratt R. G. Thorp, B. Ward, and R. J. P. Williams, *Biochem. J.*, 1970, **120**, 263.

[23] F. Nome and J. H. Fendler, *J.C.S. Dalton*, **1976**, 1212.
[24] E. W. Abel, J. M. Pratt, R. Whelan, and P. J. Wilkinson, *J. Amer. Chem. Soc.*, **1974**, **96**, 7119.
[25] R. A. Firth, H. A. O. Hill, J. M. Pratt, and R. G. Thorp, *Analyt. Biochem.*, **1968**, **23**, 429.
[26] R. Yamada, S. Shimizu, and S. Fukui, *Biochim. Biophys. Acta*, 1966, **124**, 195.
[27] G. I. H. Hanania and D. H. Irvine, *J. Chem. Soc.*, **1964**, 5694.
[28] G. C. Hayward, H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *J. Chem. Soc.* (*A*), **1971**, 196.
[29] A. W. Johnson, D. Oldfield, R. Rodrigo, and N. Shaw, *J. Chem. Soc.*, **1964**, 4080.
[30] R. O. Brady and H. A. Barker, *Biochem. Biophys. Res. Comm.*, **1961**, **4**, 373.
[31] A. W. Johnson, N. Shaw, and F. Wagner, *Biochim. Biophys. Acta*, **1963**, **72**, 107.
[32] I. P. Rudakova, E. G. Chauser, and A. M. Yurkevich, *Bioorg. Chem.*, **1975**, **1**, 616.
[33] I. P. Rudakova, T. E. Ershova, A. B. Belikov, and A. M. Yurkevich, *J.C.S. Chem. Comm.*, **1978**, 592.
[34] G. N. Schrauzer, J. A. Seck, R. J. Holland, T. M. Beckham, E. M. Rubin, and J. W. Sibert, *Bioinorg. Chem.*, **1972**, **2**, 93.
[35] C. Rüchardt, *Angew. Chem. Internat. Edn.*, 1970, **9**, 830.
[36] G. Wilkinson, *Pure Appl. Chem.*, **1972**, **30**, 627.
[37] E. A. Stadlbauer, R. J. Holland, F. P. Lamm, and G. N. Schrauzer, *Bioinorg. Chem.*, **1974**, **4**, 67.
[38] G. N. Schrauzer, L. P. Lee, and J. W. Sibert, *J. Amer. Chem. Soc.*, 1970. **92**, 2997.
[39] R. G. S. Banks, R. J. Henderson, and J. M. Pratt, *J. Chem. Soc.* (*A*), 1968, 2886.
[40] G. N. Schrauzer, *Angew. Chem. Internat. Edn.*, **1977**, **16**, 233.
[41] R. Blackburn, A. Y. Erkol, G. O. Phillips, and A. J. Swallow, *J.C.S. Faraday I*, **1974**, **1693**.
[42] P. Dowd, paper presented at the 3rd European Symposium on Vitamin B_{12} and Intrinsic Factor, Zürich, 1979.
[43] P. Dowd, M. Shapiro, and K. Kang, *J. Amer. Chem. Soc.*, **1975**, **97**, 4745.
[44] G. Bidlingmaier, H. Flohr, V. M. Kempe, T. Krebs, and J. Rétey, *Angew. Chem. Internat. Edn.*, **1975**, **14**, 822.
[45] J. Rétey, paper presented at the 3rd European Symposium on Vitamin B_{12} and Intrinsic Factor, Zürich, 1979.
[46] M. P. Atkins, B. T. Golding, and P. J. Sellars, *J.C.S. Chem. Comm.*, **1978**, 954.
[47] A. Bury, M. R. Ashcroft, and M. D. Johnson, *J. Amer. Chem. Soc.*, **1978**, **100**, 3217.
[48] S. M. Chemaly and J. M. Pratt, unpublished work.
[49] J. Halpern, *Ann. New York Acad. Sci.*, **1974**, **239**, 2.
[50] J. M. Pratt, in 'Vitamin B_{12},' ed. D. Dolphin, Wiley, New York, in the press.